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Year: 2011

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## **Comparison of manganese oxide nanoparticles and manganese sulfate with regard to oxidative stress, uptake and apoptosis in alveolar epithelial cells**

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**Abstract:** Due to their physicochemical characteristics, metal oxide nanoparticles (NPs) interact differently with cells compared to larger particles or soluble metals. Oxidative stress and cellular metal uptake were quantified in rat type II alveolar epithelial cells in culture exposed to three different NPs: manganese(II,III) oxide nanoparticles (Mn(3)O(4)-NPs), the soluble manganese sulfate (Mn-salt) at corresponding equivalent doses, titanium dioxide (TiO(2)-NPs) and cerium dioxide nanoparticles (CeO(2)-NPs). In the presence of reactive oxygen species an increased apoptosis rate was hypothesized. Oxidative stress was assessed by detection of fluorescently labeled reactive oxygen species and by measuring intracellular oxidized glutathione. Catalytic activity was determined by measuring catalyst-dependent oxidation of thiols (DTT-assay) in a cell free environment. Inductively coupled plasma mass spectrometry was used to quantify cellular metal uptake. Apoptosis rate was determined assessing the activity of caspase-3 and by fluorescence microscopic quantification of apoptotic nuclei. Reactive oxygen species were mainly generated in cells treated with Mn(3)O(4)-NPs. Only Mn(3)O(4)-NPs oxidized intracellular glutathione. Catalytic activity could be exclusively shown for Mn(3)O(4)-NPs. Cellular metal uptake was similar for all particles, whereas Mn-salt could hardly be detected within the cell. Apoptosis was induced by both, Mn(3)O(4)-NPs and Mn-salt. The combination of catalytic activity and capability of passing the cell membrane contributes to the toxicity of Mn(3)O(4)-NPs. Apoptosis of samples treated with Mn-salt is triggered by different, potentially extracellular mechanisms.

DOI: <https://doi.org/10.1016/j.toxlet.2011.05.1037>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-53950>

Journal Article

Accepted Version

Originally published at:

Frick, R; Müller-Edenborn, B; Schlicker, A; Rothen-Rutishauser, B; Raemy, D O; Günther, D; Hattendorf, B; Stark, W; Beck-Schimmer, B (2011). Comparison of manganese oxide nanoparticles and manganese sulfate with regard to oxidative stress, uptake and apoptosis in alveolar epithelial cells. *Toxicology Letters*, 205(2):163-172.

DOI: <https://doi.org/10.1016/j.toxlet.2011.05.1037>

## **Comparison of Manganese Oxide Nanoparticles and Manganese Sulfate with Regard to Oxidative Stress, Uptake and Apoptosis in Alveolar Epithelial Cells**

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## Abstract

Due to their physicochemical characteristics, metal oxide nanoparticles (NPs) interact differently with cells compared to larger particles or soluble metals. Oxidative stress and cellular metal uptake were quantified in rat type II alveolar epithelial cells in culture exposed to three different NPs: Manganese(II,III) oxide nanoparticles ( $\text{Mn}_3\text{O}_4$ -NPs), the soluble manganese sulfate (Mn-salt) at corresponding equivalent doses, titanium dioxide ( $\text{TiO}_2$ -NPs) and cerium dioxide nanoparticles ( $\text{CeO}_2$ -NPs). In the presence of reactive oxygen species an increased apoptosis rate was hypothesized. Oxidative stress was assessed by detection of fluorescently labeled reactive oxygen species and by measuring intracellular oxidized glutathione. Catalytic activity was determined by measuring catalyst-dependent oxidation of thiols (DTT-Assay) in a cell free environment. Inductively coupled plasma mass spectrometry was used to quantify cellular metal uptake. Apoptosis rate was determined assessing the activity of caspase-3 and by fluorescence microscopic quantification of apoptotic nuclei. Reactive oxygen species were mainly generated in cells treated with  $\text{Mn}_3\text{O}_4$ -NPs. Only  $\text{Mn}_3\text{O}_4$ -NPs oxidized intracellular glutathione. Catalytic activity could be exclusively shown for  $\text{Mn}_3\text{O}_4$ -NPs. Cellular metal uptake was similar for all particles, whereas Mn-salt could hardly be detected within the cell. Apoptosis was induced by both,  $\text{Mn}_3\text{O}_4$ -NPs and Mn-salt. The combination of catalytic activity and capability of passing the cell membrane contributes to the toxicity of  $\text{Mn}_3\text{O}_4$ -NPs. Apoptosis of samples treated with Mn-salt is triggered by different, potentially extracellular mechanisms.

## Keywords

Metal oxide nanoparticles, manganese, catalytic activity, apoptosis, particle uptake, type II alveolar epithelial cells.

## 1. Introduction

Metal oxide nanoparticles (NPs) represent an industrially important class of nanomaterials. Evaluation of their hazards to human health is crucial and therefore has been the purpose of numerous in vitro and in vivo studies in the past (Oberdörster et al., 2005).

The occupational environment in manganese ore processing, metallurgy and metalworking is a possible source of inhalational exposure to particulate manganese oxides. Industrial applications of manganese (Mn) are resided in manufacturing of dry cell batteries (Bader et al., 1999) and in production of organo-manganese fungicides (Ferraz et al., 1988). Methylcyclopentadienyl manganese tricarbonyl (MMT) is added to unleaded gasoline in certain countries. This leads to Mn emissions, which may be a significant source of exposure besides the occupational environment (Normandin et al., 2004). Potentially exposed occupation groups are miners, welders and smelters (Sárközi et al., 2009). It was reported that manganese is a major toxicant in welding fumes (Antonini et al., 1999, 2003, 2004; Hałatek et al., 2005). Their inhalation can be a trigger for a wide range of respiratory diseases like emphysema, lung cancer, chronic

obstructive pulmonary disease, fibrosis, asthma (Boojar and Goodarzi, 2002; Howard and Billings, 2000). Increase in frequency, duration, and severity of upper and lower respiratory tract infections and the occurrence of metal fume fever were reported (Ahsan et al., 2009; Antonini et al., 2003). Limbach et al. (2007) showed that manganese(II,III) oxide nanoparticles ( $\text{Mn}_3\text{O}_4$ -NPs) cause generation of reactive oxygen species (ROS) in human lung cancer cells (A549). However, most of the data concerning effects of manganese-NPs on the respiratory system was collected epidemiologically and little is known about their interactions on the cellular level.

Titanium dioxide nanoparticles  $\text{TiO}_2$ -NPs are used as an ingredient of sunscreens, cosmetic products, foods and medicines and they have been largely used in pulmonary toxicology studies as a negative control when assessing the toxicity of pathogenic particulates (Johnston et al., 2009). Park et al. (2008a) found decreased cell viabilities, induction of oxidative stress and increased caspase-3 activities in human bronchial epithelial cells (BEAS-2B) after exposure to  $\text{TiO}_2$ -NPs. In contrast to this, Xia et al. (2008) did not describe any adverse effects of  $\text{TiO}_2$ -NPs in the same cell line. Cerium dioxide nanoparticles ( $\text{CeO}_2$ -NPs) are used for the production of solar cells, fuel cells, gas sensors, oxygen pumps and for metallurgical and glass/ceramic applications (Lin et al., 2006). Lin et al. (2006) found cytotoxic effects and induction of oxidative stress in human lung alveolar type-II cells (A549) after exposure to  $\text{CeO}_2$ -NPs. Park et al. (2008b) additionally detected increased caspase-3 activities and induction of oxidative stress related genes in human bronchial epithelial cells (BEAS-2B). However, Xia et al. (2008) detected antioxidant and protective effects of  $\text{CeO}_2$ -NPs on BEAS-2B cells.

Glutathione is a major antioxidant of the cell, existing in two different forms: the reduced sulfhydryl form (GSH) and the oxidized disulfide form (GSSG). Reactive oxygen species (superoxide anion ( $\text{O}_2^{\cdot-}$ ), hydroxyl radical ( $\cdot\text{OH}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), hydro peroxides ( $\text{R}-\text{OOH}$ )) are neutralized by GSH in enzyme catalyzed reactions including glutathione peroxidases, glutathione-S-transferases and glutathione reductase (Rahman et al., 2006). The subsequent increase of GSSG is a sensitive marker for oxidative stress (Griffith, 1980). Generation of ROS and the subsequent increase of GSSG concentration (and the associated depletion of GSH) have been described as initiators of apoptosis (Hancock et al., 2001; Higuchi, 2004; Rahman et al., 2006; Simon et al., 2000). Donaldson et al. (1996, 2009) suggested that the response to nanoparticles could be seen as a response to oxidative stress, leading to anti-oxidant defense, proinflammatory signaling and induction of apoptosis.

The aim of this study was to determine oxidative stress and cellular metal uptake in rat type II alveolar epithelial cells (AEC) exposed to  $\text{Mn}_3\text{O}_4$ -NPs and soluble manganese sulfate (Mn-salt). Results were compared with the according effect of  $\text{TiO}_2$ - and  $\text{CeO}_2$ -NPs. We hypothesized that due to their oxidative character  $\text{Mn}_3\text{O}_4$ -NPs would induce apoptosis in comparison to dissolved Mn-salt. In addition we postulated that  $\text{TiO}_2$ - and  $\text{CeO}_2$ -NPs would not exhibit a similar effect as the  $\text{Mn}_3\text{O}_4$ -NPs. To exclude size dependent effects, NPs with a similar size and specific surface area but different in their physicochemical characteristics were chosen.

## 2. Materials and Methods:

## 2.1. Chemicals

Unless otherwise stated, chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA). Caspase-3 substrate was purchased from Merck Chemicals (Nottingham, UK). HBSS, sodium bicarbonate, sodium pyruvate, F-12 Nutrient Mixture and DMEM was purchased from Invitrogen (Basel, CH).

## 2.2. Nanoparticle preparation and characterization

Nanoparticles were prepared by flame spray synthesis (Athanassiou et al., 2010; Loher et al., 2005; Mädler et al., 2002) and thoroughly characterized for their size distribution, specific surface area and crystalline phase (Stark, 2011). Further information about synthesis and characterization is provided in the supplementary information (SI; Protocol S1).

## 2.3. Alveolar epithelial cells and exposure to NPs

Type two AEC (CCL-149), purchased from the American Type Culture Collection, Manassas, VA, USA, represent a clonally isolated cell line from the adult rat lung (Douglas and Kaighn, 1974; Madjdpour et al., 2000). Cells were maintained in F-12 Nutrient Mixture (Ham), supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin and 0.66% (v/v) sodium bicarbonate (7.5%) and grown at 37°C in a 5% CO<sub>2</sub> humidified environment. Passages 8-20 were used for the experiments. Cells were plated into 6-well (TPP, Trasadingen, Switzerland), 24-well (Corning Inc., Corning, NY, USA) and 96-well plates (Thermo Fisher Scientific, Rochester, NY, USA), according to the assay. Samples for transmission electron microscopy were prepared with cell culture inserts (surface area of 4.2 cm<sup>2</sup>, pores with 3.0 µm in diameter, high pore density PET membranes for six-well plates; BD Falcon, BD Biosciences, Basel, Switzerland). Fluorescence microscopic assays were performed in 8-well chamber slides (Thermo Fisher Scientific, Rochester, NY, USA). Cells were cultivated until monolayer reached more than 95% confluence. Culture medium was changed to assay medium (DMEM, supplemented with 1% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin and 1% (v/v) sodium pyruvate 100 mM) 16 hours prior to experiments. Nanoparticle-stock solutions of 200 parts per million (ppm, corresponding to µg particles per ml assay medium) were diluted to the final exposure concentrations in assay medium. The final exposure concentrations of NPs were 5 ppm, 10 ppm and 20 ppm. Exposure volumes were 100 µl (96-well), 300 µl (24-well and 8-well chamber slides), 2.5 ml (6-well and cell culture inserts) per well. In order to create a control setting in which cells were exposed to the same amounts of manganese in its ionized form, Mn-salt at concentrations of 11.1 ppm, 22.2 ppm and 44.3 ppm were used (corresponding equivalent doses to 5 ppm, 10 ppm and 20 ppm Mn<sub>3</sub>O<sub>4</sub>-NPs).

## 2.4. ROS-Assay

Measurement of oxidation of non-fluorescent 2,7'-dichlorofluorescein (H<sub>2</sub>DCF) substrate to the highly fluorescent form of the dye (DCF) was shown to be a sensitive method to detect generation of ROS (Foucaud et al., 2007; Wang and Joseph, 1999). After exposure to NPs, cells were washed once with HBSS and subsequently loaded with 50 µM 2,7'-dichlorofluorescein diacetate (H<sub>2</sub>DCFDA) over 30 minutes,

followed by a second washing step. Afterwards cells were fixed in 4% paraformaldehyde, pH 7.4, for 15 minutes and once again washed with HBSS. Samples were embedded in Dako Fluorescence Mounting Medium (Dako, Glostrup, DK) and subsequently visualized by a Leica LX fluorescence microscope using a regular FITC fluorescence filter and a 20x (multi immersion, NA 0.7) objective. The same microscope settings (exposure time and gain) were used for all samples. Pictures were taken with a Leica DFC 350 FX, cooled fluorescence black and white camera and qualitatively analyzed.

## 2.5. Determination of total and oxidized glutathione

Measurements of glutathione were performed using the method of Vandeputte et al. (1994) with minor modifications influenced by Tietze (1969), Allen et al. (2001), Griffith (1980) and Rahman et al. (2006). The assay is based on the oxidation of reduced glutathione (GSH) by the sulfhydryl reagent 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5-thio-2-nitrobenzoic acid (TNB), measurable spectrophotometrically. The disulfide formed (GS-TNB) is recycled to GSH by glutathione reductase in the presence of NADPH. The rate of the reaction is proportional to the concentration of total glutathione (GSX). In order to determine the amount of oxidized glutathione (GSSG), aliquots of the same samples are treated with 2-vinylpyridine prior to the assay, which covalently reacts with GSH (but not GSSG). A detailed assay protocol is provided in the supplementary information (SI; Protocol S2, Fig. S1).

## 2.6. Protein Assay

The total protein concentration was measured with a BioRad DC Protein Assay Kit (BioRad, Hercules, CA), following the manufacturer's protocol. Bovine serum albumin was used for standards.

## 2.7. DTT-Assay

Catalytic activity was determined using the method of Cho et al. (2005). Redox-active compounds catalyze the reduction of oxygen to superoxide by dithiothreitol (DTT), which is oxidized to its disulfide. The remaining thiol reduces DTNB to TNB which is determined by its absorbance. The DTT consumption indicates the catalytic activity of NPs in the samples. Nanoparticles at concentrations of 1.25 ppm, 2.5 ppm, 5 ppm, 10 ppm, 20 ppm and Mn-salt at corresponding equivalent doses were incubated at 37°C with 100 µM DTT in 0.1 M potassium phosphate buffer at pH 7.4. After one hour, 200 µl of trichloroacetic acid 10% was added to 200 µl of each of the reaction mixtures. Aliquots of 100 µl were transferred into 96-well assay plates and 200 µl of Tris-HCL 0.4 M, pH 8.9, containing EDTA 20 mM and 5 µl of 10 mM DTNB was added. Absorbance of formed TNB was read at 405 nm.

## 2.8. Inductively coupled plasma mass spectrometer

After exposure to NPs, cells were washed three times with potassium- and calcium-free phosphate-buffered solution as described by Limbach et al. (2005). Cells were transferred into PTFE digestion vessels by rinsing the wells once with 1 ml nitric acid (HNO<sub>3</sub>) and a second time with 0.5 ml HNO<sub>3</sub>. After adding 0.2 ml of H<sub>2</sub>O<sub>2</sub> (30 %, suprapur, Merck, Darmstadt), the vessels were digested in a microwave digestion unit (Ultraclave II, Milestone Inc., Shelton USA) at a pressure of up to 50 bars and 200 °C. The solutions were

then transferred into pre-cleaned polypropylene containers (15 ml test tubes, Sarstedt AG) and diluted with high purity water (Millipore SA, France) to a final weight of approximately ten g. Before analysis all samples were diluted 100 times by weight and an internal standard (Rh, final concentration 5 ng/g) was added. Elemental analysis of the solutions was carried out using an inductively coupled plasma mass spectrometer (ICPMS, 7500cs, Agilent Technologies, Palo Alto, USA) using standard operating conditions. External calibration using aqueous standards, prepared from 1000 mg/l single element standards in 1% (w/w) nitric acid. Because of the small sample size, no statistical analysis was performed.

## 2.9. Transmission electron microscopy (TEM)

Sample preparation was performed as described by Rothen-Rutishauser et al. (2005). Briefly, cell cultures were fixed in 2.5% glutaraldehyde in 0.03 M potassium phosphate buffer, pH 7.4. After post-fixation with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, the samples were stained with 0.5% uranyl acetate in 0.05 M maleate buffer. Cells were then dehydrated in a graded series of ethanol and embedded in Epon. Ultra-thin (less than or equal to 90 nm) sections were cut, mounted on coated copper grids and post-stained with uranyl acetate and lead citrate (if required for increasing contrast). Specimens were observed with a Morgagni TEM (FEI, Eindhoven, Netherlands), operated at 80 kV and equipped with a Morada camera (Gloor Instruments AG, Uster, Switzerland). Image processing was performed with the AnalySIS iTEM software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

## 2.10. Caspase-3 Assay

Caspase-3 activity was determined by measuring proteolytic cleavage of the fluorogenic caspase-3 substrate Ac-Asp-Glu-Val-Asp-AMC, as previously described by Z'graggen et al. (2010). After exposure to NPs, cells were washed with HBSS, treated with a lysis buffer containing 2.5% (v/v) HEPES 1 M, 1% (v/v) magnesium chloride 0.5 M, 1% (v/v) DTT 0.1 M, 0.1% (v/v) Triton X-100 and distilled H<sub>2</sub>O. Afterwards, cells were incubated for 1 h at 37°C with 10 µM caspase-3 substrate. Fluorescence of the cleaved reporter group was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm.

## 2.11. TUNEL Assay (TdT-mediated dUTP-X nick end labeling)

As verification for the caspase-3 results, fluorescence microscopic assays were run using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Rotkreuz, Switzerland), following the manufacturer's protocol. The TUNEL reaction preferentially detects DNA strand breaks generated during apoptosis by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction. After TUNEL-labeling, we additionally performed a 4',6-diamidino-2-phenylindole (DAPI) staining (Roche, Rotkreuz, Switzerland), as a nuclear counter stain (DAPI, 1 µg/ml in phosphate buffered saline (PBS) for ten minutes, followed by three washing steps in PBS). Afterwards, cells were embedded in Dako Fluorescence Mounting Medium (Dako, Glostrup, DK) and subsequently visualized by a Leica LX fluorescence microscope using regular DAPI and FITC fluorescence filters and a 20x (multi immersion, NA 0.7) objective. Pictures were taken with a Leica DFC 350 FX, cooled fluorescence black and white camera, in a uniform random sampling mode, generating at

least nine different pictures per chamber. Staurosporin at a concentration of 1  $\mu$ M was used as positive control. Quantitative image analysis was performed with Imaris (Bitplane Inc. Saint Paul, MN, USA).

## 2.12. Lactate Dehydrogenase Assay

Cytotoxicity was assessed by measurement of lactate dehydrogenase (LDH) released into the culture supernatants of damaged cells, using the CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay (Promega, Madison, USA), following the manufacturer's protocol.

## 2.13. Statistical analysis

Statistical analysis was performed with a one-way analysis of variance (ANOVA) with Bonferroni correction. Kolmogorov-Smirnov test was used for testing for normality of the distribution. Groups in the DTT-Assay were compared with the independent-samples T-Test. Calculations were performed using Excel for Windows and SPSS for Windows (SPSS Inc., Chicago, IL, USA). P-values less than 0.05 were considered to be statistically significant.

# 3. Results:

## 3.1. Characterization of NPs

Primary particle diameter was calculated as surface area equivalent diameter (dBET) to be within the range of 30 nm. In suspension, the mean hydrodynamic diameter (dhydr) was approximately 100 nm. Manganese oxide nanoparticles were in the hausmannite phase and titanium dioxide nanoparticles consisted of a mixture of anatase and rutile. Identity of cerium dioxide nanoparticles was confirmed. Further information on particle characteristics is provided in the supplementary information (SI; Table S1, Fig. S2, S3, S4).

## 3.2. Oxidative Stress in AEC

Interaction of all three NPs with regard to ROS production was evaluated. Cells exposed to Mn<sub>3</sub>O<sub>4</sub>-NPs (5 ppm, 10 ppm and 20 ppm) exhibited a higher H<sub>2</sub>DCFDA-fluorescence intensity compared to cells exposed to Mn-salt (44.3 ppm) (Fig. 1), indicating an increased generation of ROS. Upon exposure to TiO<sub>2</sub>-NPs and CeO<sub>2</sub>-NPs no oxidation of H<sub>2</sub>DCFDA could be detected (SI; Table S2).

To determine the redox balance of the cells, extra- and intracellular glutathione was measured. Extracellular GSSG levels increased time-dependently in cells exposed to Mn<sub>3</sub>O<sub>4</sub>-NPs, as well as in untreated cultures (Fig. 2a). After 24 hours, Mn<sub>3</sub>O<sub>4</sub>-NPs-exposed cells showed a significant higher value compared to controls ( $p < 0.001$ ). Intracellular GSSG levels, however, reached a plateau after 12 hours of exposure to Mn<sub>3</sub>O<sub>4</sub>-NPs, while exposure to medium alone did not cause any increase in GSSG levels (Fig. 2b).

As shown in Fig. 3a extracellular GSSG levels augmented by 30% (20 ppm Mn<sub>3</sub>O<sub>4</sub>-NPs) and 26% (44.3 ppm Mn-salt), compared to untreated cultures. The increase of extracellular GSSG levels did not differ significantly between cells exposed to Mn<sub>3</sub>O<sub>4</sub>-NPs and cells exposed to Mn-salt. Intracellular GSSG levels



increased by 802% (20 ppm  $\text{Mn}_3\text{O}_4$ -NPs) and 20% (44.3 ppm Mn-salt) after 24 hours, where the increase upon Mn-salt was not significant (Fig. 3b). For the amount of total glutathione (GSX) (reduced and oxidized glutathione) a significant upregulation of 26% (20 ppm  $\text{Mn}_3\text{O}_4$ -NPs) and 72% (44.3 ppm Mn-salt) after 24 hours (Fig 3c) was observed. No significant changes in glutathione contents were detected upon exposure to  $\text{TiO}_2$ -NPs and  $\text{CeO}_2$ -NPs (SI; Fig. S5).

### 3.3. Catalytic activity (DTT-Assay)

Contents of DTT in samples loaded with  $\text{Mn}_3\text{O}_4$ -NPs were reduced to 84.1% (1.25 ppm), 63.2% (2.5 ppm), 41.9% (5 ppm), 40.8% (10 ppm) and 37.2% (20 ppm) after one hour of incubation. Corresponding equivalent doses of Mn-salt did not significantly reduce DTT contents (Fig. 4).  $\text{TiO}_2$ - and  $\text{CeO}_2$ -NPs showed no significant catalytic activity at the three concentrations (SI; Fig. S6).

### 3.4. Determination of intracellular NPs and salt

Figure 5 shows intracellular contents of manganese in cells exposed to  $\text{Mn}_3\text{O}_4$ -NPs and Mn-salt at corresponding equivalent doses for 4 and 24 hours. Cellular uptake of manganese in cells exposed to Mn-salt was detected in much smaller amounts compared to uptake in cells exposed to  $\text{Mn}_3\text{O}_4$ -NPs. The three exposure concentrations of  $\text{Mn}_3\text{O}_4$ -NPs and equivalent doses of Mn-salt correspond to manganese masses of 7.2  $\mu\text{g}$ , 14.4  $\mu\text{g}$  and 28.8  $\mu\text{g}$  per well. After 4 hours, the measured intracellular contents of manganese were 1.41  $\mu\text{g}$ , 3.84  $\mu\text{g}$  and 8.09  $\mu\text{g}$  per well (after exposure to 5 ppm, 10 ppm and 20 ppm  $\text{Mn}_3\text{O}_4$ -NPs) and 0  $\mu\text{g}$ , 0  $\mu\text{g}$  and 0.64  $\mu\text{g}$  per well (after exposure to 11.1 ppm, 22.2 ppm and 44.3 ppm Mn-salt). After 24 hours, contents were 2.71  $\mu\text{g}$ , 5.57  $\mu\text{g}$  and 11.03  $\mu\text{g}$  per well (5 ppm, 10 ppm and 20 ppm  $\text{Mn}_3\text{O}_4$ -NPs) and 0  $\mu\text{g}$ , 0.04  $\mu\text{g}$  and 1.22  $\mu\text{g}$  per well (11.1 ppm, 22.2 ppm and 44.3 ppm Mn-salt). Cellular uptake of  $\text{TiO}_2$ -NPs and  $\text{CeO}_2$ -NPs was similar to uptake of  $\text{Mn}_3\text{O}_4$ -NPs. (SI; Fig. S7).

Intracellular localization and agglomerate status was investigated with transmission electron microscopy. Nanoparticle agglomerates were found mainly surrounded by membranes. Manganese oxide nanoparticles (Fig. 6a) were present in much smaller agglomerates within the cells compared to  $\text{TiO}_2$ -NPs (Fig. 6b) and  $\text{CeO}_2$ -NPs (Fig. 6c). This can be due to the solubility of  $\text{Mn}_3\text{O}_4$ -NPs in aqueous solutions.

### 3.5. Induction of caspase-3 activity and TUNEL-reaction

Based on all the previous results apoptosis assays were performed with the hypothesis that cell death is induced by intracellular  $\text{Mn}_3\text{O}_4$ -NPs through production of reactive oxygen species. Relative caspase-3 activity showed a similar increase for all three concentrations of  $\text{Mn}_3\text{O}_4$ -NPs and their equivalent doses of Mn-salt (Fig. 7). A clearly dose-dependent effect could not be detected. TUNEL-staining showed a dose-dependent increase of apoptotic cells in samples exposed to  $\text{Mn}_3\text{O}_4$ -NPs (Fig. 8b). Cells exposed to equivalent doses of Mn-salt showed a similar increase of apoptotic cells. TUNEL-positive cells were 1.8% (Control), 6.5% (5 ppm), 9.4% (10 ppm), 12.8% (20 ppm) and 14.1% (Mn-salt 44.3 ppm), where the increase upon  $\text{Mn}_3\text{O}_4$ -NPs 20 ppm and Mn-salt 44.3 ppm was significant. Between  $\text{Mn}_3\text{O}_4$ -NPs 20 ppm and Mn-salt 44.3 ppm no significant difference could be observed. In cells exposed to  $\text{TiO}_2$ -NPs and  $\text{CeO}_2$ -NPs

no apoptosis could be observed (SI; Fig. S8 and S9). Staurosporin is known to induce apoptosis in different cells types and was used as positive control (Berkova et al., 2005; Chae et al., 2000).

### 3.6. Cytotoxicity

Release of LDH could neither be detected upon exposure to NPs nor to Mn-salt (data not shown).

## 4. Discussion:

Uptake of  $\text{Mn}_3\text{O}_4$ -NPs led to intracellular oxidative stress and apoptosis in AEC, whereas Mn-salt, which hardly passed the cell membrane did not cause oxidation of glutathione, but induced a similar apoptosis rate compared to  $\text{Mn}_3\text{O}_4$ -NPs.

Since inhalation and deposition in the pulmonary system is a potential exposure scenario for NPs, type II AEC were chosen as target cells for this study. The here used CCL 149 cells form a confluent epithelial monolayer in culture and have many biological characteristics of type II pneumonocytes of intact rat lung, such as osmiophilic lamellar bodies (Douglas and Kaighn, 1974). Exposure concentrations were chosen in a range in which no release of LDH of AEC to cell culture medium could be detected.

Oxidative stress might be induced by catalytically active properties of the nanoparticle surface (Donaldson et al., 1996; Oberdörster et al., 2005) or by transition metals released from particles (Brunner et al., 2006; Studer et al., 2010). According to Aust et al. (1985) transition metals like manganese participate in the following the reaction:  $\text{Mn}^{2+} + \text{O}_2 \rightarrow \text{Mn}^{3+} + \text{O}_2^{\cdot-}$ . The consecutive reaction of superoxide ( $\text{O}_2^{\cdot-}$ ) is catalyzed by superoxide dismutase  $2\text{O}_2^{\cdot-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$ . Hydrogen peroxide decomposes, leading to an increase of GSSG:  $2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O}$  (catalyzed by glutathione peroxidase). Another possible fate of  $\text{H}_2\text{O}_2$  is the formation of hydroxyl radicals in the presence of metal ions (Fenton or Haber-Weiss reactions):  $\text{Mn}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Mn}^{3+} + \text{OH}^- + \cdot\text{OH}$  (Aust et al., 1985; Beyersmann and Hartwig, 2008; Hancock et al., 2001). Furthermore, transition metals might oxidize GSH directly:  $2\text{Mn}^{3+} + 2\text{GSH} \rightarrow 2\text{Mn}^{2+} + \text{GSSG}$  (Donaldson and Tran, 2002).

In agreement with the results of Limbach et al (2007) Mn-salt caused less ROS generation compared to  $\text{Mn}_3\text{O}_4$ -NPs at corresponding equivalent doses (Fig. 1). All cell types are capable of exporting GSH and GSSG in order to maintain redox balance (Deneke and Fanburg, 1989). To evaluate the redox balance of our samples precisely, we determined the concentration of GSSG and total glutathione in the intracellular compartment and the concentration of GSSG in the extracellular compartment (cell culture medium). The significant increase of extracellular GSSG of cells exposed to  $\text{Mn}_3\text{O}_4$ -NPs compared to unexposed cells (Fig 2a) is indicating the attempt of the cells to keep the redox balance by exporting GSSG to the extracellular compartment. Intracellular GSSG concentration increased time-dependently in the samples exposed to  $\text{Mn}_3\text{O}_4$ -NPs after 3-4 hours (Fig. 2b). Upon exposure to Mn-salt no significant increase of GSSG was observed (Fig. 3b). Surprisingly, the concentration of total glutathione was increased after 24 hours of exposure to  $\text{Mn}_3\text{O}_4$ -NPs and even more upon exposure to Mn-salt (Fig. 3c). This might be due to an upregulated synthesis of GSH by the gamma-glutamylcysteine synthetase or to an elevated activity of the

transport system for the amino acids cystine and glutamate, which serve as substrates for the de novo synthesis of GSH. Deneke and Fanburg (1989) showed that concentrations of GSH in lung cells can be significantly elevated 24 hours after induction of oxidative stress. The mechanism by which Mn-salt caused this effect without oxidizing intracellular glutathione remains unknown. Taken together the exclusive illustration of GSH without showing the relation between total and oxidized glutathione holds the possibility of an underestimation of oxidative stress in the cell.

Limbach et al. (2007) showed that both,  $\text{Mn}_3\text{O}_4$ -NPs and Mn-salt are capable of oxidizing DCF to similar amounts in a cell free system. We measured the ability of NPs and Mn-salt to catalyze the transfer of electrons from DTT to  $\text{O}_2$ , generating superoxide anions ( $\text{O}_2^{\cdot-}$ ) in a cell-free environment. Only  $\text{Mn}_3\text{O}_4$ -NPs exhibited catalytic activity in this reaction (Fig. 4). Manganese-salt oxidizes DCF or other reduced compounds, but does not participate as a catalyst in the DTT-reaction.

As shown in figure 5, uptake of  $\text{Mn}_3\text{O}_4$ -NPs seemed to be time- and dose-dependent whereas intracellular manganese in cells exposed to Mn-salt was hardly detectable. Manganese ions were predominantly kept back;  $\text{Mn}_3\text{O}_4$ -NPs have passed the plasma membrane. Verma and Stellacci (2010) summarized that polar molecules such as ions and NPs are incapable of passing the plasma membrane on their own. Ions are transported across the cell membrane by specialized membrane-transport protein channels, whereas NPs are endocytosed and dispose in endolysosomes. The intracellular dissolution within lysosomes is followed by a disruption of lysosomes with a subsequent release of metals or by generating radicals inside the cell (Studer et al., 2010). This mode of uptake has been termed Trojan horse type mechanism (Limbach et al., 2007).

In figure 6, representative TEM images show agglomerated particles surrounded by membranes. Titanium dioxide nanoparticles and cerium dioxide nanoparticles were found in solid, circumscribable agglomerates (Fig. 6b and 6c), whereas  $\text{Mn}_3\text{O}_4$ -NPs were found exclusively in very small bulks (Fig 6a). This observation might be explained by the fact that  $\text{TiO}_2$ -NPs and  $\text{CeO}_2$ -NPs are insoluble (Brunner et al., 2006), whereas  $\text{Mn}_3\text{O}_4$ -NPs are partially soluble in aqueous solutions (Limbach et al., 2007).

Cells exposed to Mn-salt exhibited an apoptosis pattern similar to that of cells exposed to  $\text{Mn}_3\text{O}_4$ -NPs, even though their redox balance was not impaired (Fig. 7) (Table 1). The findings of the TUNEL-assays were consistent with the caspase-3 results (Fig. 8). Apoptosis seemed to be triggered in a multi factorial way in our study and not only by impairment of the redox balance. An explanation might be that divalent manganese ions can pass through plasma membranes via cation transporters and bind to biological ligands of opposite charge, such as amino acid side chains of proteins and phosphate groups of nucleotides and nucleic acids (Beyersmann and Hartwig, 2008). As we showed, the uptake of metal ions occurs only in very small amounts compared to nanoparticle uptake and is therefore as a major factor less probable. Another potential toxicity mechanism was proposed by Vanwinkle et al. (2009). Several transition metal ions as copper and manganese transfer electrons to  $\text{O}_2$ , leading to  $\text{H}_2\text{O}_2$ -generation in the extracellular compartment, what might give rise to a  $\text{H}_2\text{O}_2$ -induced damage of the cell exterior. This mechanism might be a reason for the fact that Mn-salt caused an increased concentration of total

intracellular glutathione and an increased apoptosis rate without increasing the intracellular GSSG concentration.

Figure 9 illustrates the interactions of  $\text{Mn}_3\text{O}_4$ -NPs and manganese ions with AEC and points out the difference between nanoparticle surface mediated and dissolution effects as proposed for copper-NPs by Studer et al. (2010). Furthermore, potential pathways of oxidative stress formation are summarized (refers to the literature cited above). Partially soluble  $\text{Mn}_3\text{O}_4$ -NPs might act via both, surface mediated and dissolution effects. Manganese-salt dissolves within the medium and manganese ions predominantly remain in the extracellular compartment (cell culture medium), whereas an accumulation on the cell membrane cannot be excluded. The mechanism by which extracellular manganese ions harm AEC is hypothesized on the right side of Fig. 9. As proposed by Vanwinkle et al. (2009) electron transfer from extracellular transition metals to  $\text{O}_2$  causes formation of superoxide anions ( $\text{O}_2^{\bullet-}$ ). Subsequent extracellular  $\text{H}_2\text{O}_2$  generation might lead to a damage of the cell exterior.

As mentioned above the impact of  $\text{TiO}_2$ -NPs and  $\text{CeO}_2$ -NPs on lung epithelial cells is discussed controversially in the literature. The reason why  $\text{TiO}_2$ -NPs and  $\text{CeO}_2$ -NPs did not induce oxidative stress and did not increase the apoptosis rate in our experimental setting might be partially due to the choice of the target cell type. Tumor derived cells might be more metabolically active than the here used cell line. Furthermore, it has been shown that the biological responses upon exposure to  $\text{TiO}_2$ -NPs depend on the crystalline phase of the particles: The anatase phase caused greater toxicities compared to the rutile one (Johnston et al., 2009; Sayes et al., 2006; Wang et al., 2008). The composition of our  $\text{TiO}_2$ -NPs (mixture of anatase and rutile) might therefore have had a different effect on AEC. This indicates the importance of a thorough physicochemical characterization prior to the experiments.

In conclusion  $\text{Mn}_3\text{O}_4$ -NPs and Mn-salt interact with the glutathione equilibrium in different ways. These effects can be made obvious by illustrating the total and the oxidized fraction of glutathione. Although  $\text{Mn}_3\text{O}_4$ -NPs and Mn-salt do not exert the same impact on the redox balance of the cells and even with different cellular uptake, the effects on the apoptosis rates are similar. Nanoparticles incapable of oxidizing GSH did not induce apoptosis even with an uptake comparable with  $\text{Mn}_3\text{O}_4$ -NPs. Further investigations have to be performed to reveal the mechanisms by which extracellularly disposed manganese ions harm alveolar epithelial cells.

## Acknowledgement

Excellent technical assistance was provided by Christa Booy, Melanie Hasler, Livia Reyes, Birgit Roth-Z'graggen and Mohammed Ouanella. We thank Ludwig K. Limbach for particle characterization.

This work was financially supported by private funding, administered by UBS, Zurich, Switzerland.

## Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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